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13. ABSTRACT (Maximum 200 Words) Although <i>Cripto</i> was the first member of the EGF-CFC gene family to be identified, its biochemical function has been poorly understood. The function of <i>Cripto</i> was initially of interest because its overexpression was found in approximately 80% of human breast carcinomas, as well as in colorectal and pancreatic cancers. Recent genetic data from mouse and zebrafish studies indicates that EGF-CFC proteins function as co-factors or co-receptors for Nodal, a member of the transforming growth factor-beta family. In my ongoing work, I have expressed soluble Nodal and Cripto proteins in conditioned media from transfected mammalian cells, and have used a co-immunoprecipitation approach to detect a binding interaction between Nodal and EGF-CFC proteins. These biochemical studies should contribute to understanding the molecular mechanism of Cripto activity in mammary development and tumorigenesis.			
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Introduction:

Cripto encodes an extracellular protein that is a member of the *EGF-CFC* gene family. Previous studies have suggested that *Cripto* may be involved in human breast carcinogenesis (Salomon et al. 1999), particularly since *Cripto* is consistently overexpressed in approximately 80% of human breast cancers, but not in adjacent normal breast tissue (Qi et al. 1994). Other studies have shown that *Cripto* displays transforming activity when overexpressed in NOG-8 mouse mammary epithelial cells (Ciardiello et al. 1991). In addition, experiments using refolded Cripto peptide have shown that Cripto can induce tyrosine phosphorylation of the SH2 adapter protein Shc in HC-11 mammary epithelial cells, resulting in increased association of Shc with Grb2 and elevated MAPK kinase activity (Kannan et al. 1997). To elucidate the potential role of *Cripto* in breast development and tumorigenesis, I have been investigating the molecular mechanisms of Cripto protein action, with the objective of identifying a putative Cripto receptor(s).

Body:

Review of recent work in the field:

In contrast to previous models for Cripto protein as a growth factor (Salomon et al. 1999), recent evidence from molecular genetic studies in our lab and others indicates that EGF-CFC proteins act as essential co-factors for Nodal protein. The *Nodal* gene encodes a signaling factor that is a divergent member of the TGF-beta superfamily, and displays a mutant phenotype similar to that for *Cripto* (Zhou et al. 1993; Conlon et al. 1994). Genetic evidence also suggests that Nodal signals through Activin receptors and the Smad2 pathway (Collignon et al. 1996; Oh and Li 1997; Gu et al. 1998; Nomura and Li 1998; Waldrip et al. 1998). However, there is no biochemical evidence at present to show that Nodal protein directly interacts with Activin receptors or Cripto.

These studies have led to the proposal that Nodal and EGF-CFC proteins are inactive by themselves, while in combination their activity is equal to that of Activin (Gritsman et al. 1999). Furthermore, the phenotypes of both *Cripto* and *Cryptic* knock-out mice can be readily interpreted as resulting from defects in *Nodal* signaling (Ding et al. 1998; Yan et al. 1999). These data can be summarized in terms of a possible regulatory pathway for *Nodal* and *EGF-CFC* activities (Fig. 1). In this case, the EGF-like growth factor activities documented for Cripto protein might represent cross-talk between EGF receptor and SMAD2 signaling pathways (de Caestecker et al. 1998; Kretzschmar et al. 1999).

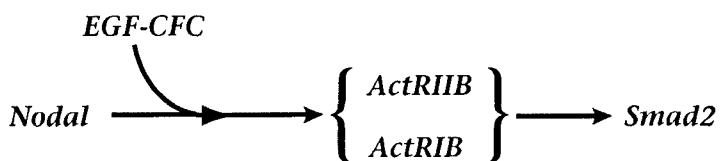


Figure 1. Potential regulatory role of *EGF-CFC* proteins in the *Nodal* signal transduction pathway. Nodal signals through Activin receptors and the Smad2 pathway but requires EGF-CFC family proteins as co-factors. It is important to note, however, that this pathway does not exclude the possibilities that EGF-CFC proteins could act as co-factors for other members of the TGF-beta superfamily, or could also act through an unrelated signaling pathway.

Technical Objective I: Expression patterns of *Cripto* and *Cripto* receptor

Strategy: In my original grant application, I proposed to survey the distribution of endogenous *Cripto* expression as well as Cripto-alkaline phosphatase (AP) fusion protein binding

sites in normal mammary tissue obtained from virgin, pregnant, lactating, and involuting mice. In particular, since it had been shown that human Cripto protein (hCripto) can bind with high affinity to a range of mammary epithelial cell lines, including MDA-MB-453, SK-BR-3 human breast cancer cells and normal mouse HC-11 cells (Brandt et al. 1994; Kannan et al. 1997), I hypothesized that these cell lines expressed putative Cripto receptors.

Results: To examine the expression of *Cripto* at various stages of mammary development, I employed RT-PCR and ribonuclease protection assays to analyze mammary glands from virgin, pregnant, lactating, and regressing stages. As described in my previous annual report, these results indicated that *Cripto* is expressed at extremely low levels throughout mammary development, with slightly elevated expression during pregnancy and lactation. In addition, I found that the binding sites detected for Cripto-AP fusion proteins on HC-11 mammary epithelial cells are likely to correspond to heparan sulfate proteoglycans, and not to signal-transducing receptors. Therefore, I have not pursued further AP-Cripto binding studies on mammary tissue, as originally proposed, but instead have focused on biochemical studies of Cripto binding interactions. Thus, my studies have addressed Task 1 of Technical Objective I in the original Statement of Work, but the lack of evidence for authentic receptor interactions do not support further studies in several lines of MMTV transgenic mice (Task 2 in the Statement of Work).

Technical Objective II: Cloning and characterization of the *Cripto* receptor

Strategy: In my original proposal and Statement of Work, I described a methodology to clone putative Cripto receptors using AP-fusion protein reagents to screen expression libraries. However, since current genetic evidence indicates that Cripto is required for Nodal function and may represent a co-receptor for Nodal, I have decided to focus on the analysis of Cripto interacting proteins using a co-immunoprecipitation approach. In particular, I have undertaken to produce Cripto and Nodal proteins in cell culture, and examine the biochemical interactions between Cripto and Nodal proteins.

Results: Previous studies have shown that zebrafish Oep is a secreted, membrane associated protein. Its association with the cell membrane is mediated by the C-terminal hydrophobic domain since C-terminal truncated Oep can be released from cell membrane (Zhang et al. 1998). To determine if this is the same case for Cripto, we performed cellular fractionation experiments, using insect cells infected with baculoviruses expressing a secreted Cripto protein fused at its N-terminus to glutathione-S-transferase (GST) (Fig. 2). By Western blotting using a specific anti-Cripto antiserum, I found that that purified plasma membrane fractions contained both full-length and C-terminal truncated forms of the GST-Cripto fusion protein. In contrast, the conditioned media from infected cells contained high levels of the truncated GST-Cripto protein, but very low levels of the full-length protein. These results suggested that like Oep, full-length Cripto is also a membrane associated protein, and C-terminal truncated Cripto proteins can be secreted into culture supernatants.

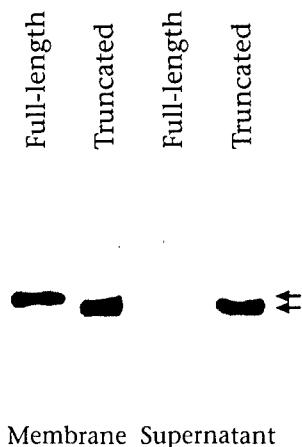


Figure 2. Detection of Cripto protein in plasma membrane fractions and culture supernatants prepared from baculovirus-infected insect cells by Western blotting using anti-Cripto antiserum. Arrows at right indicate positions of full-length and truncated GST-Cripto fusion proteins. For expression of GST-Cripto fusion proteins, cDNAs corresponding to a full-length or C-terminal truncated Cripto were cloned into the *pAcSecG2T* transfer vector (Pharmingen), which contains a *gp67* signal sequence for high-level secretion, and generates an N-terminal fusion with GST. Baculovirus stocks were prepared in Sf9 insect cells and used to infect High-5 insect cells (Invitrogen). Plasma membrane fractionation was performed essentially as described (Ozols 1990; Storrie and Madden 1990). The absence of contaminating endoplasmic reticulum in the plasma membrane fractions was confirmed using assays for cytochrome b₅ reductase.

To study Nodal protein function, I have made plasmid constructs to express secreted processed Nodal protein in mammalian cells. For this purpose, I have made two constructs, one for expression of a wild-type epitope-tagged Nodal protein, and a second for a chimeric Nodal protein with a heterologous BMP-4 prodomain. I have found that both wild-type and chimeric Nodal proteins can be secreted and processed by 293T cells. Thus, while Nodal protein is not processed successfully in transfected COS cells (data not shown), a cleaved product of the expected size can be found in the culture supernatant of transfected 293T cells (Fig. 3). This may reflect differential expression of the specific pro-protein convertase(s) required for Nodal processing (Constam and Robertson 1999).

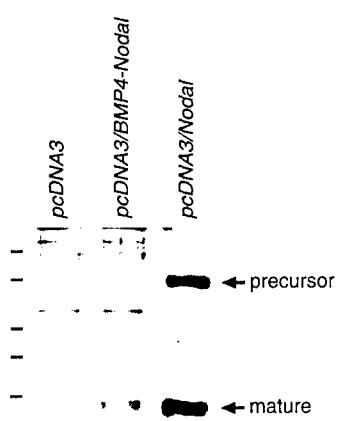
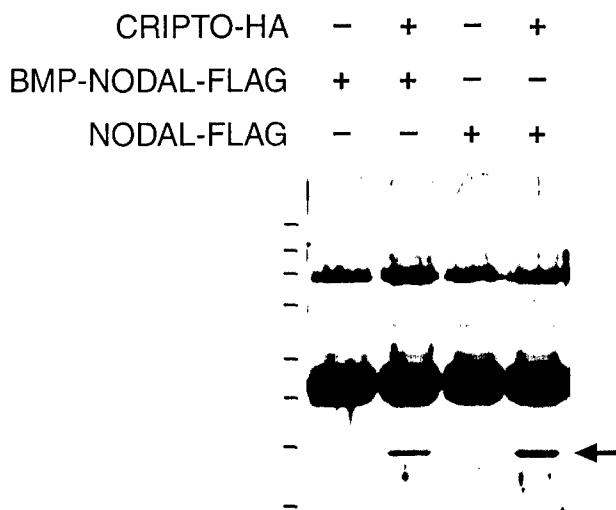


Figure 3. Preparation of processed Nodal protein in mammalian cells. Plasmid constructs that encode a chimeric BMP4-Nodal and a full-length wild-type Nodal with a FLAG tag at their C-terminus were used to transfect 293T cells. Culture supernatants were collected, concentrated 10-fold and used for Western blotting with a FLAG monoclonal antibody (Sigma). Positions of size standards at 50.3, 37.6, 25.4, 20, 14, and 9.3 kDa are indicated.

In order to detect interaction between Cripto and Nodal proteins, I performed co-immunoprecipitation assays using epitope-tagged Cripto and Nodal proteins produced in culture supernatants of transfected 293T cells. In the experiment shown in Fig. 4, I incubated HA-tagged Cripto protein with FLAG-tagged Nodal protein (either as a chimeric BMP-Nodal or a unmodified wild type Nodal) in solution, and immunoprecipitated with anti-HA monoclonal antibody-coupled beads. Western blotting using an anti-FLAG monoclonal antibody showed that processed mature Nodal protein can be co-immunoprecipitated together with Cripto protein.

Figure 4. Co-immunoprecipitation assay for interaction between Cripto and Nodal proteins. Cripto-HA and Nodal-FLAG proteins were produced in culture supernatants of transiently transfected 293T cells and were concentrated 8-fold. After incubation, protein complexes were immunoprecipitated by anti-HA monoclonal antibody-coupled beads (Covance), the beads washed 4 times with 1x PBS/0.1% NP-40, then immunoprecipitated complexes were resolved on a 12% SDS-PAGE gel and Western blotted using an anti-FLAG monoclonal antibody (Sigma). Positions of size standards at 81.6, 61.5, 50.8, 37.6, 25.4, 20, 14, and 9.3 kDa are indicated.



My experiments have provided preliminary evidence for a direct binding interaction between Cripto and Nodal. I am currently extending these results by investigating the interaction of Nodal protein with other members of the EGF-CFC family, and with various mutant forms of Cripto. I am also investigating whether a Nodal-Cripto protein complex is able to bind to a soluble extracellular domain of ActRIIB, and are attempting to establish a cell culture assay for Nodal activity using published assays for Activin activity. In summary, although I have not pursued the specific experiments proposed in Tasks 1 and 2 of Technical Objective II in the original Statement of Work, I have successfully identified a Cripto-interacting protein (Task 3 of Technical Objective II in the Statement of Work).

Key Research Accomplishments:

- I have expressed soluble Cripto and Nodal proteins in conditioned media from transfected mammalian cell lines for analyses of their activities and potential biochemical interactions.
- Co-immunoprecipitation analyses of these proteins has provided evidence of a direct binding interaction.
- My findings raise the possibility that *Cripto* overexpression in human breast cancer may result in de-regulation of TGF-beta signaling pathways.

Reportable Outcomes:

Published manuscripts:

None.

Meeting abstracts:

Shen, M. M., E, C., Saplakoglu, U., Yan, Y.-T., and Ding, J. (2000). Functional analysis of EGF-CFC genes in mouse development indicates their essential role in Nodal signaling. Era of Hope Department of Defense Breast Cancer Research Program Meeting. (Atlanta, GA).

E, C., Saplakoglu, U., and Shen, M. M. (2000). Role for Cripto as a cofactor in the Nodal signaling pathway. Era of Hope Department of Defense Breast Cancer Research Program Meeting. (Atlanta, GA).

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Conclusions:

In the past year, important progress has been made in understanding of the molecular basis for Cripto function. Previous studies have suggested that Cripto has growth factor activity, and that addition of exogenous Cripto protein to mammary epithelial cells lines can result in tyrosine phosphorylation of the ErbB4 receptor and activation of the Ras/MAPK signaling pathway. However, since Cripto does not bind to any of the four known members of the *ErbB* receptor family, it has been unclear how Cripto might function as a ligand to activate receptor-mediated signaling. Thus, it has been significant that recent genetic data from mouse and zebrafish studies indicates that EGF-CFC proteins function as co-factors or co-receptors for Nodal.

In my work, I have expressed soluble Nodal and Cripto proteins in conditioned media from transfected mammalian cells to detect a physical interaction between Nodal and EGF-CFC proteins. My data support a direct association between Nodal and Cripto *in vivo* to facilitate Nodal signaling, presumably through activin receptors. At present, these data do not allow us to distinguish whether Cripto acts as a co-factor or as a co-receptor for Nodal; however, my ongoing studies should be able to resolve this critical issue. Finally, these biochemical studies should contribute to understanding the molecular mechanism of Cripto activity in mammary development and tumorigenesis.

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